

Ketoconazole Inhibition of Testicular Secretion of Testosterone and Displacement of Steroid Hormones from Serum Transport Proteins

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In vivo perfusion of canine testes with ketoconazole inhibited the stimulation of testosterone production by human chorionic gonadotropin in a dose-dependent manner. Ketoconazole also selectively displaced steroids from serum-binding globulins. Dihydrotestosterone and estradiol binding to sex hormone-binding globulin were inhibited by ketoconazole. Cortisol binding to corticosteroid-binding globulin was unaffected. The concentrations of ketoconazole that inhibited human chorionic gonadotropin stimulation of testicular androgen production and displaced sex steroids from sex hormone-binding globulin were in the range of blood levels found in patients on higher therapeutic dosage regimens. Suppression of testicular testosterone synthesis and displacement of estrogens from sex hormone-binding globulin may decrease the androgen/estrogen ratio of the blood and contribute to the development of gynecomastia that has been reported in some ketoconazole-treated patients.

Ketoconazole, an orally administered antifungal agent, has recently been approved for use against a variety of systemic mycoses. An apparent advantage of this drug is that its usual side effects have been minor and its major toxicities infrequent (7). However, recent reports have raised concern with regard to hormonal perturbations. De Felice et al. (4) noted an unexpected reaction, i.e. gynecomastia, in 3 of 36 males receiving the drug. Pont et al. (8a) have reported a transient reduction in serum testosterone levels in humans several hours after ketoconazole administration. These investigators also reported that ketoconazole suppressed testosterone production in rat Leydig cells in vitro (8a). In a related report, human volunteers given ketoconazole displayed a blunted cortisol response to adrenocorticotropin, and ketoconazole interfered with adrenal stimulation in vitro (9). These studies suggest that ketoconazole may have a generalized inhibitory effect on steroid-secreting tissues. The relationship between inhibition of steroidogenesis and gynecomastia is not clear since it appears that an increase in free estrogen is also required for gynecomastia to develop (17).

The physiological activity of circulating steroid hormones is modulated by means of binding proteins in the blood with specificities directed

toward each class of hormones (16). The biological potency of gonadal steroids has been demonstrated to be inversely related to the fraction present in the serum bound to sex hormone-binding globulin (SHBG) (12, 15). Metabolic conversion and clearance rates for androgens appear to be more closely related to the free steroid levels than to their total serum concentrations (14). Ketoconazole is highly protein bound (1) and therefore might interfere with steroid binding to SHBG. A decrease in binding of testosterone to SHBG would be expected to enhance testosterone clearance from the circulation and thus could contribute to the fall in blood levels observed after ketoconazole treatment. Such displacement phenomena might also be of significance in regard to the development of gynecomastia. In view of these early reports on the influence of ketoconazole on endocrine activity, we have investigated further the effects of this drug on endocrine function.

MATERIALS AND METHODS

Reagents. Estradiol-(2,4,6,7,16,17-³H)(N) (137.1 Ci/mmol), dihydrotestosterone (DHT)-(1,2-³H) (50 Ci/mmol), testosterone-(1,2-³H)(N) (50.4 Ci/mmol), and hydrocortisone-(1,2-³H)(N) (50.7 Ci/mmol) were obtained from New England Nuclear Corp., Boston,

Mass. Each tritium-labeled steroid was analyzed for purity by thin-layer chromatography on a silica gel and found to be at least 97% pure. Unlabeled estradiol, DHT, diethylstilbestrol, cortisol, and chorionic gonadotropin (hCG) were obtained from Sigma Chemical Co., St. Louis, Mo. Ketoconazole (molecular weight, 531.43, lot no. C2101) was provided by Janssen Pharmaceutica, Inc., New Brunswick, N.J. Other reagents and chemicals were of analytical grade quality.

Testicular perfusion. Six male greyhound dogs, weighing 24 to 45 kg each, were used. Each dog was anesthetized with sodium pentobarbital (30 mg/kg of body weight) to allow the testes perfusion. The method for perfusion of the isolated testes in vivo has been previously described in detail (2). Briefly, this involves isolating both testes, cannulating their vascular supply, returning them to the scrotum, and perfusing the testes with the animal's own blood. To eliminate variations in blood flow to the testes, a peristaltic pump (model 375A; Sage Instrument Div., Orion Research Inc., Cambridge, Mass.) was used to deliver blood at a rate of 3.87 ml/min in all perfusions. This rate of perfusion has been previously shown to equal the normal blood flow through dog testes (2). Ketoconazole was acidified with 0.2 N HCl and mixed with normal dog plasma for maximum solubility. Control plasma was prepared by addition of an equimolar quantity of 0.2 N HCl to that present in the plasma to which ketoconazole had been added. One group of dogs ($n = 3$) received an infusion of ketoconazole calculated to give a blood concentration of 5 $\mu\text{g/ml}$, and a second group of dogs ($n = 3$) was infused with ketoconazole at a level calculated to give a blood concentration of 20 $\mu\text{g/ml}$.

In each experiment, the testes were perfused with blood alone for 30 min before ketoconazole or hCG infusions were begun. Ketoconazole was infused into one spermatic artery for 150 min while the contralateral control testis was infused with control plasma. After 60 min of control plasma or ketoconazole infusion, an infusion of hCG at a rate of 0.075 IU/min was begun to both spermatic arteries of each animal. This rate has previously been found to stimulate testosterone secretion submaximally in this model (2). The control plasma, ketoconazole, and hCG infusions were discontinued at 150 min, after which all testes were perfused with blood alone for an additional 30 min. After 180 min of perfusion, each spermatic artery was injected with 25 IU of hCG to assess the viability of the preparation. During the study, spermatic venous effluent was collected in test tubes and centrifuged at 4°C, and the plasma was stored frozen (-70°C) until assayed for testosterone.

Testosterone was measured by radioimmunoassay as previously described (2). Ketoconazole in concentrations of up to 60 $\mu\text{g/ml}$ was not found to interfere with the testosterone assay. Spermatic venous plasma testosterone responses to infused hCG are expressed as the area under the testosterone curve (60 to 150 min) above the unstimulated spermatic venous plasma testosterone concentration. Unpaired t tests were used for statistical analysis.

Relative binding. Pooled serum from normal adult human males was treated with 50 mg of activated charcoal per ml (Norit A; Pfanstiehl Laboratories Inc., Milwaukee, Wis.) at 23°C for 1 h to remove endogenous steroids. No detectable steroids remained in the

stripped serum as determined by radioimmunoassay (2, 6). The treated serum was stored at -20°C.

(i) **DHT and estradiol.** Steroid binding to serum proteins was determined by differential ammonium sulfate precipitation to separate bound from free hormone (8, 11). Serum was diluted 1:10 with 0.01 M sodium phosphate buffer (pH 7.6) containing 0.15 M sodium chloride. The diluted serum was incubated for 1 h at 23°C with either 2×10^{-10} M [^3H]DHT or 1×10^{-10} M [^3H]estradiol. At the end of the preincubation period, 0.25 ml of serum was transferred to glass tubes (12 by 75 mm) preequilibrated to 4°C, to which 0.25 ml of buffer containing various amounts of nonlabeled displacer, 10^{-11} to 10^{-4} M in ethanol, had been added. Within each experiment, each concentration of displacer was run in at least triplicate. Owing to the low solubility of ketoconazole in aqueous solution, all compounds used for displacement studies were dissolved in and diluted to appropriate concentrations with absolute ethanol. The final ethanol concentration in all tubes, including saline controls, was 1% (vol/vol). The incubation was then continued for 6 h at 4°C in the presence of displacer, at which time the tubes were placed in an ice bath. After 10 min, 0.5 ml of a 100% saturated solution of ammonium sulfate was added with mixing. The tubes remained in the ice bath for an additional 10 min and were then centrifuged at $1,900 \times g$ for 30 min. Samples of 0.5 ml of the supernatant were transferred to glass vials, and the radioactivity was counted in a model 3133 liquid scintillation spectrometer (Beckman Instruments, Inc., Irvine, California), using a commercial scintillant (Aquamix; West Chem Products, North Hollywood, Calif.) at an efficiency of 39% as determined by internal standard.

(ii) **Cortisol.** Serum diluted 1:10 with phosphate buffer was incubated with 10^{-10} M [^3H]cortisol for 1 h at 23°C. Displacement of [^3H]cortisol from corticosteroid-binding globulin was assessed as described above for DHT and estradiol, with the following exceptions: (i) 0.2 ml of serum preincubated with [^3H]cortisol was added to 0.2 ml of buffer containing displacer, and (ii) 1 ml of 100% ammonium sulfate was added at the end of the 6-h incubation period.

Relative binding was calculated with respect to the binding of tritium-labeled steroid in the absence of any unlabeled steroid and is reported as the mean \pm standard error of the mean.

Ketoconazole analysis. Serum concentrations of ketoconazole were determined as described previously, utilizing an agar well diffusion assay (3). Briefly, ketoconazole hydrochloride was added to blood group AB, heat-inactivated serum to construct a standard curve for each plate on which serum samples were run. The indicator organism employed was *Kluyveromyces fragilis* 55-1. The sensitivity of this assay is 0.05 $\mu\text{g/ml}$.

Concentrations of ketoconazole in human semen were determined in a similar fashion, except semen from the same individual before ketoconazole administration was used in preparation of the standards.

RESULTS

Testicular perfusion. The effect of ketoconazole on testicular testosterone production was evaluated by measuring the response of the in

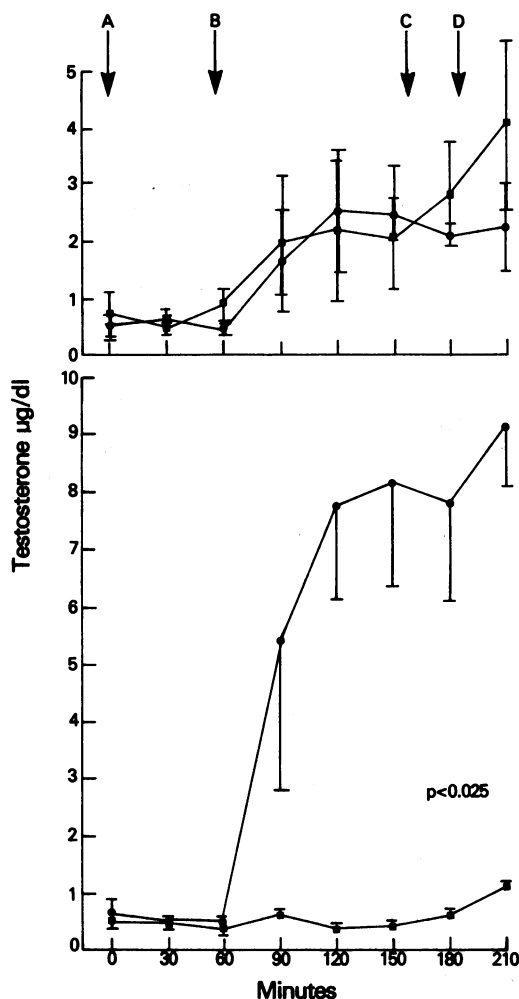


FIG. 1. Spermatogenic venous testosterone concentration (mean \pm standard error) during perfusion of isolated canine testis in vivo. Control testes received control plasma, whereas low-dose dogs (top) received 5 μ g of ketoconazole per ml and high-dose dogs (bottom) received 20 μ g of ketoconazole per ml. The testes were perfused with blood from 0 to 210 min. At 0 min (A), ketoconazole infusions were started for treated testes (■) and control plasma infusions were started for the control testes (●). At 60 min (B), hCG (0.075 IU/min) was added to both infusates. All infusions were terminated at 150 min (C), and 30 min later (D), hCG (25 IU) was injected into the spermatic artery of all testes.

vivo perfused dog testis to hCG stimulation. Each dog was used as its own control. Two concentrations of ketoconazole (5 and 20 μ g/ml) were studied in separate groups of three dogs each. Ketoconazole had no effect on either basal or hCG-stimulated testosterone production by the testes at the lower drug concentration (5 μ g/ml) used in these studies (Fig. 1). At the high

dose level (20 μ g/ml), there was no effect on basal testicular production of testosterone. However, there was a marked impairment in the hCG-stimulated response (control, 377.5 ± 102.8 μ g \cdot min/dl, as compared with ketoconazole, 1.08 ± 8.1 μ g \cdot min/dl; $P < 0.025$). All of the testes exhibited a response to the hCG injection at the conclusion of the perfusion period. No differences in arterial or venous pH were found between control dogs (arterial, 7.35 ± 0.02 ; venous, 7.34 ± 0.03 [mean \pm standard deviation]) or treated dogs (arterial, 7.35 ± 0.02 ; venous, 7.32 ± 0.03).

Plasma concentrations of ketoconazole are shown in Table 1. There was general agreement between the calculated and measured concentrations except in the higher concentration range, owing possibly to an overestimation of drug content (13) by the bioassay. The arterial-venous differences in ketoconazole concentrations for all animals demonstrate a substantial decrease of the measured drug in venous as compared with arterial samples.

Penetration of ketoconazole into the human genitourinary tract. In view of the findings of in vivo suppression of testosterone synthesis in humans and dogs and the degree of ketoconazole extraction demonstrated in the canine perfusion studies, it was of interest to determine whether ketoconazole is present in the human genitourinary tract. This was assayed by determining semen ketoconazole concentrations in a patient receiving 400 mg daily for scrotal coccidioidomycosis. Samples taken at 1 and 3 h after administration of the drug yielded ketoconazole concentrations of 0.90 and 0.25 μ g/ml, respectively.

Displacement of steroids from serum-binding proteins. The ability of ketoconazole to compete with gonadal steroids for binding to SHBG is demonstrated by the displacement of [3 H]DHT (Fig. 2). Inspection of the displacement curves revealed that the affinity of ketoconazole toward SHBG was substantially lower than that of either DHT or estradiol and approximates that of

TABLE 1. Plasma ketoconazole concentration in dog spermatic arteries and veins during isolated testes perfusion

Dog	Dose (μ g/ml)	Concn (μ g/ml) in:	
		Arteries	Veins
1	5	7.4	4.0
2		4.3	0.4
3		8.3	3.1
4	20	44.0	21.0
5		35.0	11.5
6		14.5	4.6

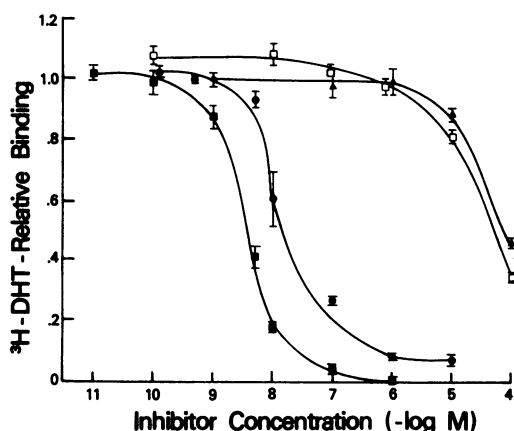


FIG. 2. Inhibition of [3 H]DHT binding to SHBG. Diluted serum was incubated with 0.2 nM [3 H]DHT with and without inhibitor. SHBG-bound hormone was precipitated by 50% ammonium sulfate after 6 h of incubation at 4°C. Samples of supernatant were counted, and bound hormone was determined by the difference from the total [3 H]DHT added. Relative binding was calculated with respect to binding of [3 H]DHT in the absence of added unlabeled steroid. ■, DHT; □, ketoconazole; ▲, diethylstilbestrol; ●, estradiol. Data represent a composite of values obtained in two separate experiments.

diethylstilbestrol. The concentration of ketoconazole necessary to displace 50% of the bound [3 H]DHT was 4×10^{-5} M, as compared with 3.5×10^{-9} M for DHT itself or 15×10^{-9} M for estradiol (Table 2). The effect of ketoconazole on the binding of [3 H]estradiol to SHBG was also evaluated (Table 2); the results obtained were similar to those obtained when [3 H]DHT was used as the binding probe. The order of effectiveness of the compounds studied as displacing agents in both cases was DHT > estradiol > ketoconazole ~ diethylstilbestrol. The con-

centrations of ketoconazole needed to displace 50% of the DHT and estradiol bound to SHBG were 21 and 10 μ g/ml, respectively. The concentrations of each of the displacers necessary to reduce DHT binding by 50% were higher than those required to achieve the same degree of displacement of estradiol binding. This observation is consistent with the greater affinity of SHBG reported for androgens than for estrogens (5, 10). The greater concentration of ketoconazole required to displace DHT relative to that needed to displace estradiol is consistent with the differential affinity of SHBG toward these two steroids.

In contrast to the results of studies of the displacement of gonadal steroids from SHBG, ketoconazole had no effect on binding of [3 H]cortisol to corticosteroid-binding globulin even at a concentration of 10^{-4} M (Table 2). Cortisol reduced binding of itself by 50% at 5×10^{-8} M. At 10^{-5} M DHT, cortisol binding was reduced by only 20%. No measurable displacement of cortisol was observed when diethylstilbestrol was used at 10^{-4} M.

DISCUSSION

In perfused canine testes, ketoconazole impaired hCG-stimulated testosterone production when infused at a concentration of 20 μ g/ml; in contrast, there was no apparent inhibition at a concentration of 5 μ g/ml. Basal testosterone production rates were unaffected by ketoconazole infusion. Ketoconazole has also been reported to inhibit hCG-stimulated synthesis of testosterone in rat Leydig cells *in vitro* (8a). In that study, a drug concentration of 5 μ g/ml blocked basal and gonadotropin-stimulated testosterone production. The reason for the greater sensitivity of the *in vitro* Leydig cell preparation may reflect differences in experimental conditions or species. The suppressive action of ketoconazole on testicular responsiveness to hCG *in vivo* would appear to be reversible, as evidenced by the enhancement of testosterone production induced by a pharmacological dose of hCG after discontinuation of drug infusion. Pont et al. (8a) also found evidence for reversibility. It therefore appears that, at least during short-term exposures to the drug, inhibition of hCG-stimulated testosterone production by the testes requires the continued presence of ketoconazole.

In our perfusion experiments, venous ketoconazole concentrations were found to be lower than arterial levels in all of the dogs studied. The observed arterio-venous differential may be attributable to accumulation of drug in testicular tissue or *in vivo* conversion to metabolites undetectable by the bioassay for ketoconazole or both. The extraction of ketoconazole from the

TABLE 2. Displacement of steroid hormones from serum-binding proteins

Displacing agent ^a	Bound steroid ^b		
	DHT	Estradiol	Cortisol
Ketoconazole	4×10^{-5}	2×10^{-5}	10^{-4}
Diethylstilbestrol	7×10^{-5}	2×10^{-5}	ND
DHT	3.5×10^{-9}	0.6×10^{-9}	1×10^{-5c}
Estradiol	15.0×10^{-9}	2.5×10^{-9}	10^{-4}
Cortisol	ND	ND	5×10^{-8}

^a Concentrations (molar) of displacing agents are those which reduced binding of labeled steroid by 50%.

^b Each value represents the mean from data obtained in two separate experiments. ND, Not determined.

^c Twenty percent displacement at 10^{-5} M.

circulation by various tissues and body fluids has been reported previously (3). The presence of ketoconazole in human semen indicates that this drug can be found in male genitourinary tract secretions as well.

In addition to the blockade of testosterone synthesis, ketoconazole may have more generalized inhibitory effects on steroidogenic tissue function. Ketoconazole has also been reported to decrease adrenal corticosteroid synthesis in response to adrenocorticotropin in humans (9). Although the clinical manifestations of adrenal insufficiency have not been reported, it remains a concern, especially with higher or multiple daily doses as those currently employed in therapeutic trials of various systemic mycoses (A. Pont, J. R. Graybill, and P. C. Craven, *Clin. Res.*, in press).

The selective displacement of steroid hormones from serum-binding globulins as shown in these studies demonstrates a previously unreported effect of ketoconazole on the endocrine system. Ketoconazole displaced 50% of the DHT binding to SHBG at a concentration of 21 $\mu\text{g/ml}$ and 50% of the estradiol binding at 10 $\mu\text{g/ml}$. In contrast, no similar displacement of cortisol from corticosteroid-binding globulin was observed. This finding provides further evidence that the decreased adrenocorticotropin-stimulated cortisol levels measured in humans (9) is due to decreased production and not to steroid displacement from corticosteroid-binding globulin.

Concentrations of ketoconazole that interfere with steroid binding to serum transport proteins in our studies are within the range of those measured in humans during treatment with ketoconazole. Serum levels of ketoconazole are highly variable among patients receiving a given dose of the drug but have been found to be as high as 20 $\mu\text{g/ml}$ at a dose of 400 mg/day and up to 50 $\mu\text{g/ml}$ at 800 mg/day (3). Furthermore, as dosage levels are increased, peak blood levels of ketoconazole persist for longer periods and, after repeated treatments, result in sustained drug levels greater than anticipated from a single administration (3). Thus, in patients on long-term therapy at high doses of ketoconazole, blood concentrations may reach levels in excess of those which were found to displace steroids from serum-binding globulins in vitro.

The mechanism by which ketoconazole induces gynecomastia remains to be determined. Our studies provide support for multiple endocrine effects of the drug which could alter the endocrine status of an individual sufficiently to cause gynecomastia. Perturbations in the ratio of blood levels of sex steroids is believed to be an important factor in the onset of gynecomastia (17). The suppression of testicular androgen production and concomitant displacement of es-

trogens from SHBG would both contribute to a modification of the blood androgen/estrogen ratio and thus satisfy one of the major criteria for the development of this condition. Further detailed investigation of free and bound hormone levels in humans will be necessary to evaluate the magnitude of the effects of ketoconazole on the circulating androgen/estrogen ratio and to what extent these effects contribute to the development of gynecomastia.

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LITERATURE CITED

1. Borelli, D., J. Bran, J. Fuentes, R. Legendre, E. Leiderman, H. B. Levine, and M. A. Restrepo. 1979. Ketoconazole, an oral antifungal agent: laboratory and clinical assessment of imidazole drugs. *Postgrad. Med. J.* 55:657-661.
2. Boyden, T. W., R. W. Pamentor, and M. A. Silvert. 1980. Testosterone secretion by the isolated canine testes after controlled infusions of hCG. *J. Reprod. Fertil.* 59:25-30.
3. Brass, C., J. N. Gaigiani, T. F. Blaschke, R. DeFelice, R. A. O'Reilly, and D. A. Stevens. 1982. Disposition of ketoconazole, an oral antifungal, in humans. *Antimicrob. Agents Chemother.* 21:151-158.
4. DeFelice, R., D. G. Johnson, and J. Gaigiani. 1981. Gynecomastia with ketoconazole. *Antimicrob. Agents Chemother.* 19:1073-1074.
5. Dunn, J. F., B. C. Nisula, and D. Rodbard. 1981. Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* 53:58-68.
6. Emmert, Y., W. P. Collins, and I. F. Sommerville. 1972. Radioimmunoassay of oestrone and oestradiol in human plasma. *Acta Endocrinol. (Copenhagen)* 69:567-582.
7. Heel, R. C., R. N. Brogden, A. Carmine, P. A. Morley, T. M. Speight, and G. S. Avery. 1982. Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. *Drugs* 23:1-36.
8. O'Connor, S., H. W. G. Baher, A. Dulmanis, and B. Gudson. 1973. The measurement of sex steroid binding globulin by differential ammonium sulfate precipitation. *J. Steroid Biochem.* 4:331-339.
- 8a. Pont, A., P. L. Williams, S. Azhar, R. E. Reitz, C. Bochara, E. R. Smith, and D. A. Stevens. 1982. Ketoconazole blocks testosterone synthesis. *Arch. Intern. Med.* 142:2137-2140.
9. Pont, A., P. L. Williams, D. S. Loose, D. Feldman, R. E. Reitz, C. Bochara, and D. A. Stevens. 1982. Ketoconazole inhibits adrenal steroid synthesis. *Ann. Intern. Med.* 97:370-372.
10. Pugeat, M. M., J. F. Dunn, and B. C. Nisula. 1981. Transport of steroid hormones: interaction of 70 drugs with testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* 53:69-75.
11. Rosner, W. 1972. A simplified method for the quantitative determination of testosterone-estradiol-binding-globulin activity in human plasma. *J. Clin. Endocrinol. Metab.* 34:983-988.
12. Stumpf, P. G., R. M. Nakamura, and D. R. Mishell. 1981. Changes in physiologically free circulating estradiol and testosterone during exposure to levonorgestrel. *J. Clin. Endocrinol. Metab.* 52:138-143.
13. Swezey, S., K. M. Giacomini, A. Abang, C. Brass, D. A.

- Stevens, and T. F. Blaschke. 1982. Measurement of ketoconazole, a new antifungal agent, by high-performance liquid chromatography. *J. Chromatogr.* 227:510-515.
14. Vermeulen, A., and S. Ando. 1979. Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. *J. Clin. Endocrinol. Metab.* 48:320-326.
15. Vermeulen, A., T. Stoica, and L. Verdonck. 1971. The apparent free testosterone concentration, an index of androgenicity. *J. Clin. Endocrinol. Metab.* 33:759-767.
16. Westphal, N. 1978. Mechanism of steroid binding to transport proteins. p. 33-47. In E. Genazzani, F. Di Carlo, and W. I. P. Mainwaring (ed.), *Pharmacological modulation of steroid action*. Raven Press, New York.
17. Wilson, J. D., and P. C. MacDonald. 1980. The pathogenesis of gynecomastia. *Adv. Intern. Med.* 25:1-32.